BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. **02-192**)

PATENT

In the Application of:	)	
	)	
Kozarov, *et al.*	)	
	)	Art Unit: 1642
Serial No.: **09/849,115**	)	
	)	Examiner: G. Nickol
Filed: **May 5, 2001**	)	
	)	
For: **Methods and Compositions for Angioproliferative Disorder Treatment**	)	

DECLARATION

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Dr. Emil Kozarov, am a named inventor of U.S. Patent Application Serial No. 09/849,115, filed on May 5, 2001 ("the Patent Application").

2. The following experiments were performed under my supervision. First, apoptosis assays were performed with VEGF-induced primary human endothelial cells (HCAEC, Cambrex Corp., East Rutherford, New Jersey), which were grown in the presence of VEGF (2 ng/ml). It has previously been demonstrated that impaired cell-matrix contact leads to apoptosis. In order to test whether *Porphyromonas gingivalis* protease-mediated detachment of endothelial cells is linked to apoptosis, a quantitative ELISA assay was used (Roche Diagnostics). MAbs against DNA and nucleosomal

histones in the cytoplasmic fraction of liberated primary HCAEC cell lysates were used to evaluate apoptotic cell death upon 18-hr treatment with *P. gingivalis* W83 lysine-specific and arginine-specific cysteine protease-enriched SPF (soluble protein fraction) preparation (2 mg protein/ml 50 mM Tris buffer, pH 7.5).

3. The *P. gingivalis* W83 protease-enriched SPF preparation contains *P. gingivalis* cysteine proteases gingipain R (RgpA and RgpB) and gingipain K (Kgp) (collectively referred to as "the gingipains"). RgpA and RgpB are arginine-specific cysteine proteases. Kgp is a lysine-specific cysteine protease. HA2 (HArep2), a 15-kDa subunit of an adhesion repeat (HArep), found in HagA and in gingipains has also shown proteolytic activity. *See e.g.,* Paramaesvaran et al., (1998) J. Dental Res. 77:664. The gingipains are related to HagA and HArep. These proteases all share homology. For example, HA2 is a subunit (proteolytically processed fragment) of both HagA and RgpA. PrtP is a lysine-specific and arginine-specific cysteine protease of *P. gingivalis*.

4. In this experiment, attached non-*P. gingivalis*-treated HCAEC cells were used as controls. The experiment was performed in triplicate and the mean values are presented with the standard deviation (vertical bars) (See Figure 1). After 18 hrs, *P. gingivalis* W83 cysteine protease-enriched SPF treatment increases the absorbance at 405 nm approx. 4.6 times over the control. The data suggest that in addition to causing HCAEC cell detachment from the substrate and total cell number reduction, *P. gingivalis* cysteine protease treatment results in a several-fold increase in apoptosis in treated primary endothelial cells.

5. In a second set of experiments proliferation inhibition of VEGF-induced HCAEC primary endothelial cells was examined. HCAEC cells were grown in 24-well

plate and induced by cultivation in EBM-2 medium containing 10 ng/ml VEGF. All treatments were performed in triplicate and the mean values were obtained. Cells in both states were tested, confluent (quiescent) state and non-confluent growing (log phase) state. Therefore, the experiment represented existing and actively forming tumor vessels. As controls, mock-treated wells were used (treatment with 50mM Tris buffer pH 7.5). The enzymes in this experiment were purified gingipains (gingipain R (RgpA and RgpB) and gingipain K (Kgp)) which were prepared as follows. 5.0 ml of *P. gingivalis* SPF was passed over a 10 ml Bio-Scale Q column (Bio-Rad, Hercules, Calif.) equilibrated with 50 mM Tris-HCl pH 7.5 at a flow rate of 0.5 ml/min for 20 min. The column was washed with same buffer at a flow rate of 1.0 ml/min for 50 min. Proteins were eluted with 50 mM Tris-HCl, 2M NaCl, pH 7.5 at a flow rate 2.0 ml/min for 25 min. Fractions were dialyzed against 1mM Tris-HCl pH 8.0, overnight (3 times, 4L each). The procedure is essentially according to Yun (Yun *et al.*, Infect Immun *67*, 2986-2995 (1999)) with modifications.

6. The purified gingipain preparation was added at 0.4 mg/ml, and the detached cells were collected in 24 hrs and counted using Coulter Counter Z-1. The remaining attached cells were then dispersed using 0.25% trypsin and also counted. This experiment demonstrated that purified *P. gingivalis* gingipain preparation detaches tumor growth factor-induced primary human endothelial cells thereby disrupting the endothelial cell monolayer. Furthermore, the purified gingipains are especially active against dividing endothelia, and they also disrupt intact confluent (quiescent) endothelia, which can have implications in treatment not only of growing, but of stationary tumors as well (Fig. 7).

7. The proliferation inhibition of *P. gingivalis* cysteine protease-treated VEGF-stimulated primary endothelial cells is represented by total number of cells and was compared to the number of untreated cells. Purified gingipain treatment of VEGF-induced human endothelial cells results in proliferation inhibition (Fig. 8). Reducing the total cell numbers upon endothelial layer disruption demonstrates the ability of *P. gingivalis* proteases not only to disrupt the vasculature, especially the actively growing ones, but also to trigger the cell death in the liberated cells thus eliminating them altogether.

8. In addition to the two *in vitro* experiments described above, *in vivo* experiments were performed. The purified gingipain preparation, as described above, was administered intratumorally (IT) to lung and breast cancer animal models. A polyacrylamide gel of the gingipain preparation from *P. gingivalis* strain W83, containing 3.1 μg gingipains per ml 50 mM Tris buffer, pH 7.5, is presented in Figure 6. Molecular weight marker (Dual color Precision marker, BioRad, Hercules, Calif.) band of 50 kDa is adjacent to the two main gingipain bands, migrating at 45-50 kDa as demonstrated on this typical for self-processing proteolytic enzymes gel image.

9. Two established tumor cell lines were used in strain mouse syngeneic graft models, EMT6 murine breast sarcoma (Kirkpatrick *et al.,* Anticancer Drugs *3*, 651-658 (1992)) and M109 murine lung carcinoma (Schultz *et al., In Vitro 13*, 223-231 (1977)). After the tumors reached size of ~500 mm^3, the tumors in each group of animals was injected intratumorally with 3×10 μl purified gingipain preparation (29 μg/μl). The animals were inoculated with a total of 0.87 mg gingipains. In 24 hours, the tumors were

harvested and 4 μm paraffin sections were prepared. Hematoxylin/eosin stained histology sections were obtained and the morphology of the tissue was assessed.

10. The result of the treatment is shown on Figures 2-5, stained paraffin sections, magnification ×50. Figure 2 shows untreated EMT6 breast sarcoma control. Figure 3 shows gingipain-treated EMT6. Figure 4 shows, M109 lung carcinoma untreated control. Figure 5 shows gingipain-treated M109. The morphology of both treated tumors reveal large areas of necrosis consistent with the purpose of the treatment, tumor tissue disintegration and compromising the blood supply by endothelial destruction. In contrast, control untreated tumors show no sign of necrosis.

11. I conclude that administration of cysteine proteases from *P. gingivalis* leads to tissue disintegration and tumor necrosis by compromising the blood supply, detaching the cells from their substrate, and triggering cell death in, for example, breast and lung tumor tissue.

12. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon."

Date: Feb 27, 2004

Emil Kozarov

Emil Kozarov, Ph.D.

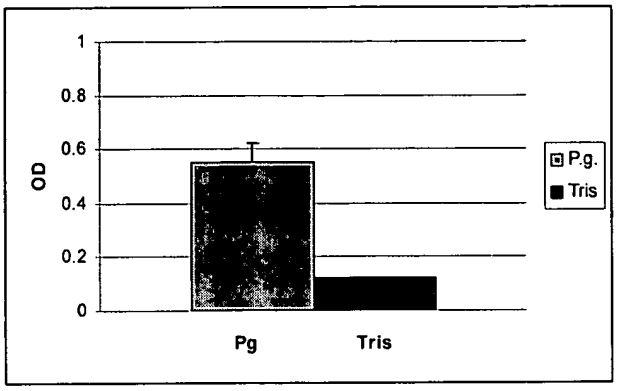

Fig. 1. Apoptosis assay

Fig. 2. EMT6 untreated control

Fig. 3. EMT6 gingipain treatment

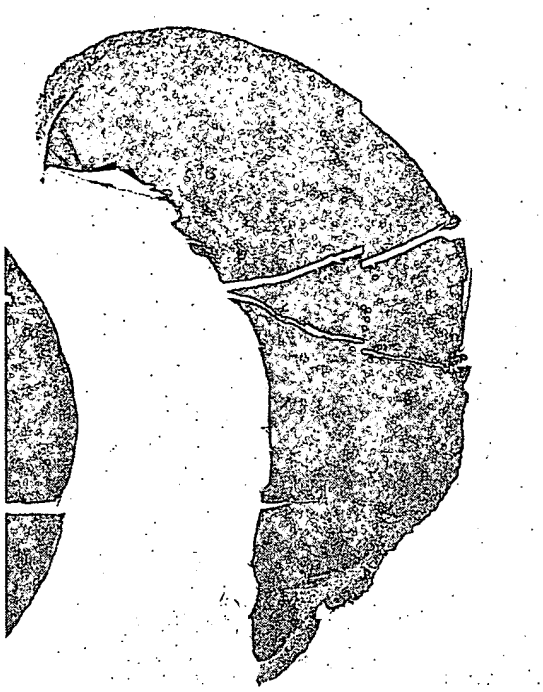

Fig. 4. M109 untreated control

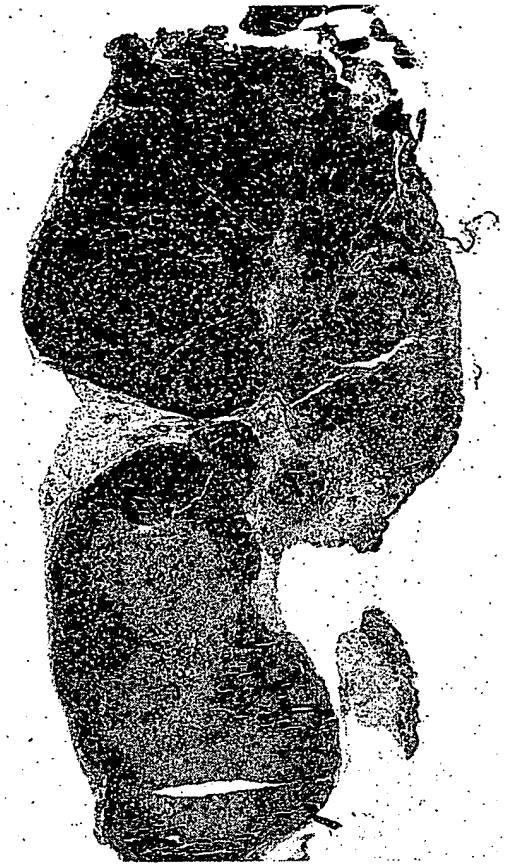

Fig. 5. M109 gingipain-treated

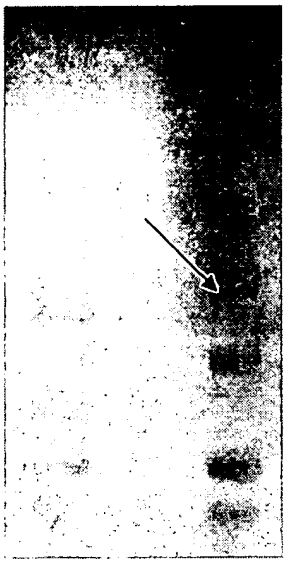

Fig. 6. Gingipain preparation. Right lane, molecular mass markers. Arrow, 50 kDa marker protein. Left lane, active gingipain ion exchanger fraction.

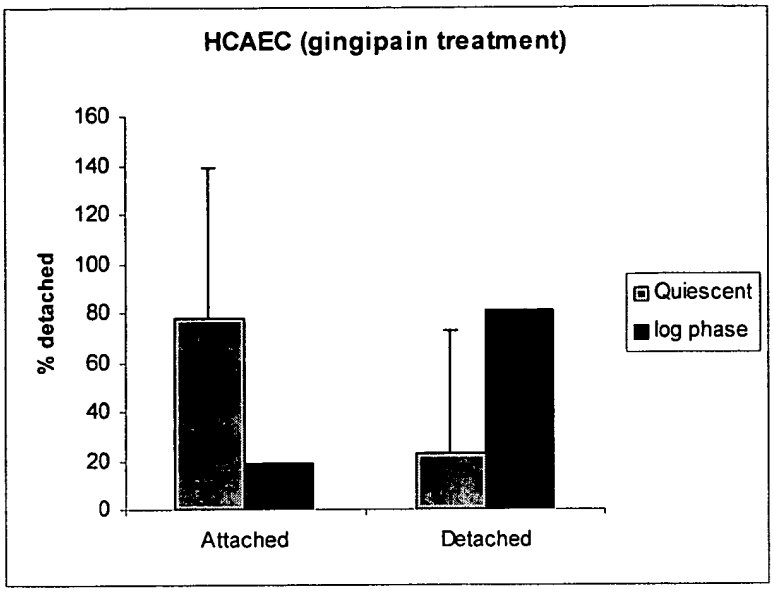

Fig. 7. Detachment of quiescent and dividing (log phase) VEGF-induced cells upon treatment with purified gingipains presented as percent of the cell number in mock-treated wells.

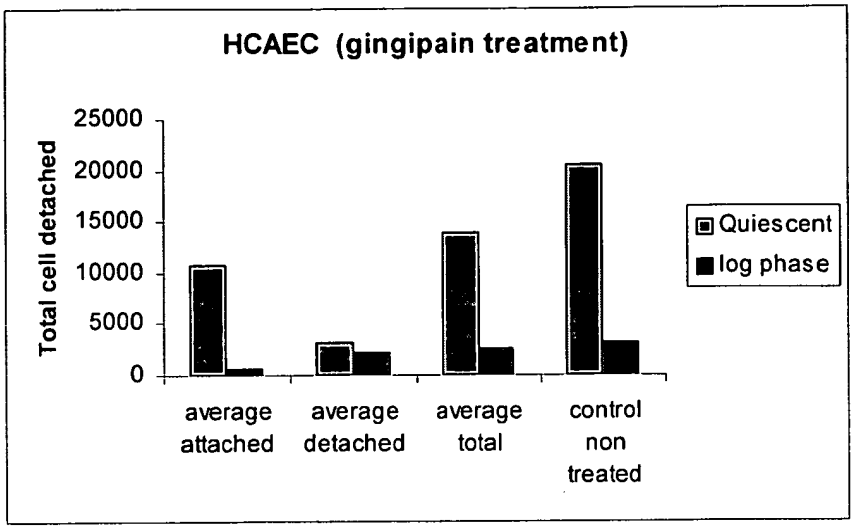

Fig. 8. Effect of gingipain treatment on proliferation of human VEGF-induced primary endothelial cells.